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Transcription factors with gradients of expression in neocortical progenitors give rise to distinct motor and sensory cortical areas by controlling the area-specific differentiation of distinct neuronal subtypes. However, the molecular mechanisms underlying this area-restricted control are still unclear. Here, we show that COUP-TFI controls the timing of birth and specification of corticospinal motor neurons (CSMN) in somatosensory cortex via repression of a CSMN differentiation program. Loss of COUP-TFI function causes an area-specific premature generation of neurons with cardinal features of CSMN, which project to subcerebral structures, including the spinal cord. Concurrently, genuine CSMN differentiate imprecisely and do not project beyond the pons, together resulting in impaired skilled motor function in adult mice with cortical COUP-TFI loss-of-function. Our findings indicate that COUP-TFI exerts critical areal and temporal control over the precise differentiation of CSMN during corticogenesis, thereby enabling the area-specific functional features of motor and sensory areas to arise.

arealization | subcerebral projection neurons | neocortex development | corticofugal neurons | nuclear receptor | behavior

The mammalian cerebral cortex, responsible for fine motor control and sensorimotor integration, is subdivided into functionally distinct areas that control motor functions and process distinct sensory modalities (1). Individual areas are distinguished by their cytoarchitecture, connectivity, physiology, and patterns of gene expression (2, 3). Each area is radially divided into six layers, and each layer consists of a variety of populations of neurons with distinctive morphologies, connectivity, and developmental programs of gene expression (4–9). In particular, layers VI and V contain corticofugal neurons, which send their axons to deep brain structures, such as the thalamus (corticothalamic neurons), the striatum (corticostriatal neurons), pons (corticopontine neurons), tectum (corticotectal neurons), and spinal cord (corticospinal motor neurons, CSMN) (7).

The fate of neurons and laminar cytoarchitecture in each specific area determines their function: the adult primary motor cortex contains a large number of CSMN and has a thick layer V; the primary somatosensory area is characterized by a thick layer IV, where the neurons that receive relayed sensory inputs are located (10). The area-specific differences in neuronal fate and cytoarchitecture have been thought to result from late postmitotic events, e.g. selective postnatal pruning of axons (11), and premitotic events, such as the timing, rate, and duration of proliferation of precursors producing distinct projection neuron subtypes (12–16). As a striking illustration of such processes, CSMN are generated at a higher rate in the developing motor cortex than in sensory areas in mice (12), but the molecular mechanisms that control this area-specific differential production of CSMN are not known. The transcription factor COUP-TFI is particularly interesting in this regard, because it is expressed at different levels in presumptive sensory and motor cortices, and could thus underlie the striking cytoarchitectural differences between these two cortical areas (17, 18). Using cortex-specific conditional loss-of-function of COUP-TFI, we have previously demonstrated that this transcription factor is critical for areal patterning by acting in sensory cortex to repress frontal/motor cortical area identity (17). COUP-TFI has also been shown to regulate neuronal differentiation (19) and, together with COUP-TFII, to control the timing of the switch of progenitor cells from neurogenesis to gliogenesis in the developing cortex (20). Given the dual function of COUP-TFI in neuronal and areal specification, we hypothesized that COUP-TFI might control sensory area formation by repressing a "motorizing" genetic program of differentiation in neurons of the somatosensory cortex.

We find this to be the case, and show that in the absence of COUP-TFI function, CSMN are born prematurely in somatosensory cortex, at a time when layer VI corticothalamic neurons are normally born. Layer V is expanded at the expense of layer VI, with a corresponding redistribution of neurons expressing CSMN-specific genes and projecting to the spinal cord. In the context of an aberrantly expanded motor cortex and a corticospinal tract consisting largely of the axons of abnormally specified corticothalamic neurons, adult *COUP-TFI* conditional mutant mice exhibit impaired fine motor skills, reinforcing the necessity for precision in both areal and temporal control of CSMN differentiation. Our results indicate a critical role for COUP-TFI in controlling the emergence of the area-specific cytoarchitectural and functional features of sensory and motor cortical areas during corticogenesis, via specific areal and temporal repression of a CSMN differentiation program in corticofugal neurons of the somatosensory cortex.

Results

COUP-TFI Regulates the Laminar Cytoarchitecture and the Molecular Identities of Corticofugal Neurons in Somatosensory Cortex. COUP-TFI is expressed in the caudal-most region of the telencephalic anlage as early as embryonic day (E) 9.5 before neurogenesis starts, and this high caudo-lateral to low rostro-medial expression pattern becomes very prominent at E13.5 during the peak period of CSMN production (Fig. S1). To investigate whether COUP-TFI might restrict the generation or specification of layer V CSMN in soma-

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tosensory cortex, we analyzed the expression of selected CSMN-specific genes in the frontal/motor (motor) and parietal/somatosensory (S1) cortices in wild type (*WT*) and in the frontal motor and parietal/somatosensory motorized (mS1) area in *COUP-TFI* conditional mutant (*CKO*) mice (Fig. 1). The transcription factors *Fezf2* and CTIP2 are specifically expressed at high levels by CSMN and related subcerebral projection neurons in layer V, and at much lower levels by corticothalamic neurons in layer VI (4, 6, 21). In *WT* mice, both *Fezf2* and CTIP2 delineate a much broader and denser layer V in the motor cortex than in S1 (Fig. 1 *A*, *C*, *D*, *G*, and *H*), reflecting area-specific differences in CSMN generation and differentiation (Fig. S2). In sharp contrast, in *COUP-TFI CKO* cortex, there is a dramatic increase in the number of high-level *Fezf2*- and CTIP2-expressing neurons in layer VI, most pronounced in mS1 and visible as an area-specific thickening of layer V (Fig. 1 *B*, *F*, and *J*). Interestingly, CTIP2 expression is strikingly increased in the most superficial layer VI neurons, called "mVI" throughout this study. We next used area-specific markers, such as *Crim1*, FOXP2, and *Igfbp4*, which, in *WT* cortex, are expressed in neurons of layer V in motor, but not in S1 cortex (4, 6) (Fig. 1 *K*, *L*, *O*, *P*, *S*, and *T*). These markers are ectopically expressed in layer V in mS1 (Fig. 1 *N*, *R*, and *V*), further confirming that loss of COUP-TFI function imparts motor-like characteristics to neurons in parietal cortex. FOXP2 and *Igfbp4* are also expressed by layer VI neurons (4) (Fig. 1 *O*, *P*, *S*, and *T*), and their expression is strikingly reduced or abolished in layer VI (Fig. 1 *Q*, *R*, *U*, and *V*), further indicating that a subset of layer VI neurons differentiate abnormally in the absence of COUP-TFI function. Abnormal cytoarchitecture is confirmed by cresyl-violet histological analysis of *COUP-TFI CKO* cortex, which shows an expansion and abnormal morphology of layer V neurons in sensory areas, and a decrease in thickness of layer VI in both sensory and motor areas of *COUP-TFI CKO* mice (Fig. S3).

Taken together, these results indicate that, in the absence of COUP-TFI function, the number of neurons expressing high levels

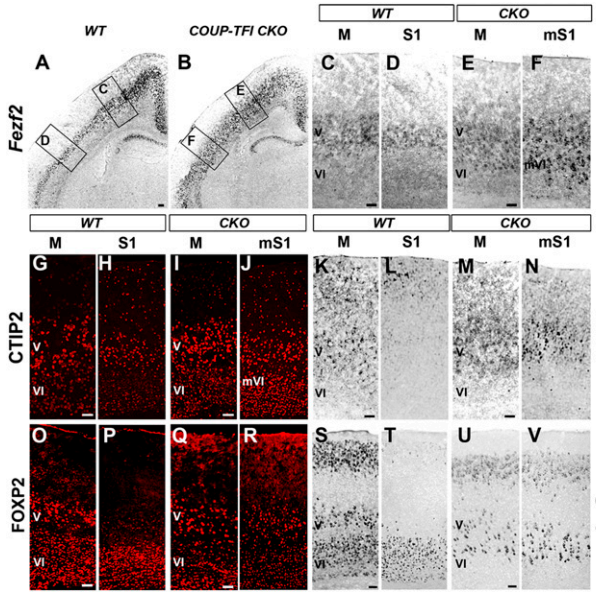

Fig. 1. Increased expression of molecular hallmarks of CSMN in corticofugal neurons of S1 cortex in *COUP-TFI CKO* mice. (*A* and *B*) Coronal sections and (*C–V*) higher magnification views of frontal (M) and parietal (S1/mS1) cortices of *WT* and *COUP-TFI CKO* P8 brains indicate abnormal expression levels of the CSMN markers *Fezf2* (*A–F*), CTIP2 (*G–J*), *Crim1* (*K–N*), FOXP2 (*O–R*), and *Igfbp4* (*S–V*) in layer V and radial expansion of these markers toward superficial layer VI (mVI) in mS1 of *COUP-TFI CKO* cortices. Note that expression of FOXP2 is reduced (*Q* and *R*) and expression of *Igfbp4* is abolished (*U* and *V*) in layer VI in both areas of *COUP-TFI CKO* cortices. [Scale bars: 50 μm (*A* and *B*); 100 μm (*C–V*).]

of CSMN markers in layer V of the motorized S1 is dramatically increased, at the expense of layer VI neurons. Importantly, in the "genuine," occipitally mislocated S1 and V1 mutant cortical areas, the pattern of CTIP2-expressing neurons is comparable to corresponding areas of *WT* animals (Fig. S4). Therefore, our data strongly suggest that COUP-TFI acts in an area-restricted manner on the differentiation of the two main classes of corticofugal neurons: corticothalamic and corticospinal motor neurons.

We next assessed expression of FOXP2, TBR1 (expressed by layer VI neurons, including corticothalamic neurons) (22), and CTIP2 (strongly expressed only in CSMN) (4), to further investigate potential interactions in the differentiation pathways of corticothalamic neurons and CSMN in the absence of COUP-TFI function (Fig. 2). In P8 *WT* mice, FOXP2/TBR1 and CTIP2 are expressed by distinct subsets of neurons in layers VI and V, respectively, with only rare layer VI and V neurons co-expressing both FOXP2 and CTIP2 or TBR1 and CTIP2 (Fig. 2 *A*, *G*, *H*, *J*, *P*, and *Q*) (6). In striking contrast, the proportion of neurons co-expressing both FOXP2 and CTIP2 (Fig. 2 *B*, *G'*, and *H'*) or TBR1 and CTIP2 (Fig. 2 *K*, *P'*, and *Q'*) is increased in layers V and VI of *COUP-TFI CKO* mice, (Fig. 2*I*) (FOXP2+CTIP2+; layer V: *WT*, 4.1 ± 1.2%; *CKO*, 15.0 ± 0.3%; $P = 0.01$; layer VI: *WT*, 13.4 ± 3.4%; *CKO*, 47.2 ± 7.8%; $P = 0.05$), (Fig. 2*R*) (TBR1+CTIP2+; layer V: *WT*, 1.3 ± 0.3%; *CKO*, 6.9 ± 1.5%; $P = 0.02$; layer VI: *WT*, 7.1 ± 0.9%; *CKO*, 21.4 ± 0.7%; $P = 0.0002$), indicating abnormal acquisition of mixed corticothalamic and CSMN identity by corticofugal neurons. Taken together, these data indicate that loss of COUP-TFI function leads to a failure of corticothalamic neurons and CSMN to differentiate along segregated molecular pathways, resulting in a large number of neurons with mixed corticothalamic and CSMN identities.

Next, we investigated the temporal course of expression of the neuron subtype-specific markers *Fezf2*, CTIP2, and TBR1 in *WT* and *CKO* cortex at E13.5 (the peak time of birth of CSMN) and at E16.5, when generation of corticofugal neurons is terminated. In the absence of COUP-TFI function, there is an expansion of the *Fezf2*- and CTIP2-positive populations at E13.5, which is matched with a reduction of the *Tbr1*-expressing cells (Fig. S5). This altered balance between *Fezf2*/CTIP2- and TBR1-positive populations is still present at E16.5, indicating that COUP-TFI is normally involved in the distinct differentiation of CSMN and corticothalamic neurons from early stages of corticogenesis, in accordance with its high expression levels in presumptive corticofugal neurons at prenatal stages (Fig. S1). Taken together, this suggests that in S1 cortex, COUP-TFI normally represses a CSMN differentiation program during generation of layer VI corticothalamic neurons and that, in the absence of COUP-TFI function, presumptive corticothalamic neurons abnormally display cardinal molecular features of CSMN differentiation.

Abnormal Expression of CSMN-Specific Genes Motorizes Layer VI Corticothalamic Neurons. We next investigated whether the abnormal expression of the transcription factors *Fezf2* and CTIP2 directs the differentiation of neurons normally destined to become corticothalamic neurons into CSMN, and results in a shift in their axonal projections to subcerebral targets instead of to the thalamus. We retrogradely-labeled subcerebrally-projecting neurons from the cerebral peduncle via ultrasound-guided microinjections of FluoroGold in P2 mice, and performed the analysis at P6 (Fig. 3 *A* and *B*). In striking contrast to *WT* mice, in which subcerebral projection neurons are sharply confined to layer V (Fig. 3 *E* and *G*), the position of these neurons in *COUP-TFI CKO* mice includes mVI of mS1, where abnormal high CTIP2-expressing neurons are located (Fig. 3 *F*, *H*, and *I"*). This finding demonstrates that abnormal expression levels of CSMN-specific control genes in presumptive corticothalamic neurons initiate central features of CSMN differentiation, including subcerebral axonal targeting. Strikingly, retrograde labeling from the spinal cord (Fig. 3*J*) reveals that these abnormal subcerebral projection

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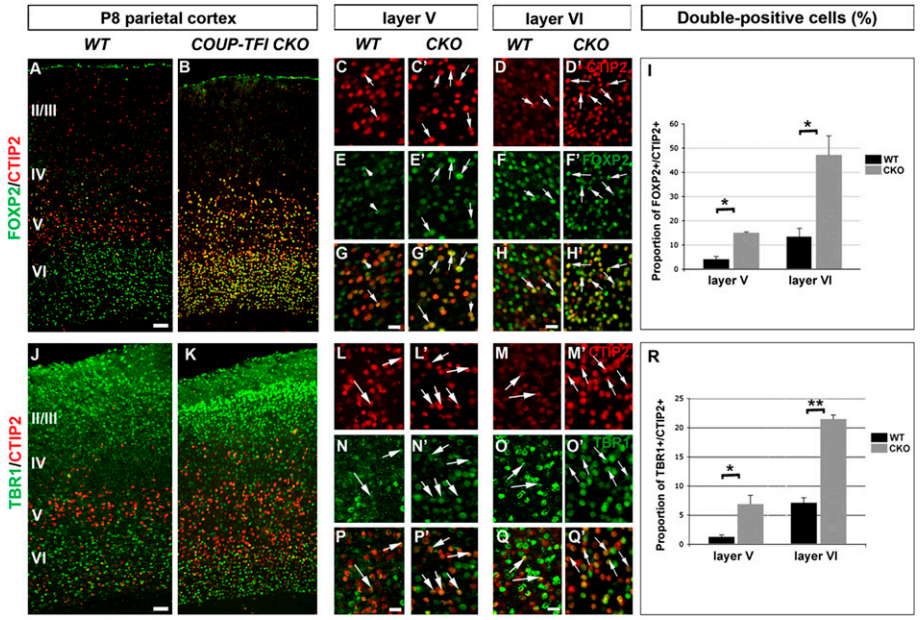

Fig. 2. Abnormal molecular specification of corticofugal neurons in *COUP-TFI CKO* parietal cortex. (*A* and *B*) Double immunofluorescence against CTIP2 and FOXP2 and higher magnification views (*C–H′*) in layers V and VI. (*J* and *K*) Double immunofluorescence against CTIP2 and TBR1 and higher magnification views (*L–Q′*) in layers V and VI. Arrows indicate neuron that co-express both markers in *WT* and *COUP-TFI CKO* P8 parietal cortices. (*I* and *R*) Quantification of number of double-positive cells per total of cells in layers V and VI indicates a significative difference between *WT* and *CKO* cortices. Error bars represent SEM. Student's *t* test, $*P \leq 0.05$, $**P \leq 0.01$. [Scale bars: 100 μm (*A, B, J,* and *K*); 20 μm (*C–H′* and *L–Q′*).]

neurons of mVI are the dominant corticofugal neuron population able to successfully send axonal projections to more caudal targets in the cervical spinal cord of *COUP-TFI CKO* mice (Fig. 3 *N* and *P″*): indeed, in layer V, genuine CSMN, which abnormally express high levels of *Fezf2*, CTIP2, FOXP2, and TBR1 (Figs. 1 and 2), send axons which reach the cerebral peduncle, but not the spinal cord. This finding indicates that transcriptional dysregulation in genuine CSMN in the absence of COUP-TFI function results in abnormal differentiation of CSMN.

Motorized Layer VI Neurons Project to Cervical, Thoracic, and Lumbar Spinal Cord. To better investigate the entire trajectories of subcerebral axons, we crossed *WT* and *COUP-TFI CKO* mutants with "*CST-YFP*" mice, which express YFP in corticofugal neurons (23) (Fig. 4). At P7, the trajectory of subcerebral projections is largely unaffected by loss of COUP-TFI function (Fig. 4 *A–M″*). However, *COUP-TFI CKO* mice develop a detectable decrease in corticolumbar projections by P21 (Fig. 4 *N* and *O* and Fig. S6), suggesting abnormal degeneration or area-specific pruning at later stages. Remarkably, this corticospinal connectivity primarily reflects axonal projections of the misspecified mVI neurons of the abnormally expanded motorized cortex, because the axons of genuine CSMN in layer V largely do not reach the cervical cord (Fig. 3 *N–P″*). Taken together, these data indicate that COUP-TFI normally controls CSMN differentiation and cortical efferent connectivity, and that loss of COUP-TFI strikingly enables a subset of late-born corticothalamic neurons to establish corticospinal projections to cervical, thoracic, and lumbar spinal cord segments.

COUP-TFI Controls the Area-Specific Timing of CSMN Specification During Genesis of Corticofugal Neurons. Our data indicate that lack of COUP-TFI predominantly affects the latest-born (i.e. most

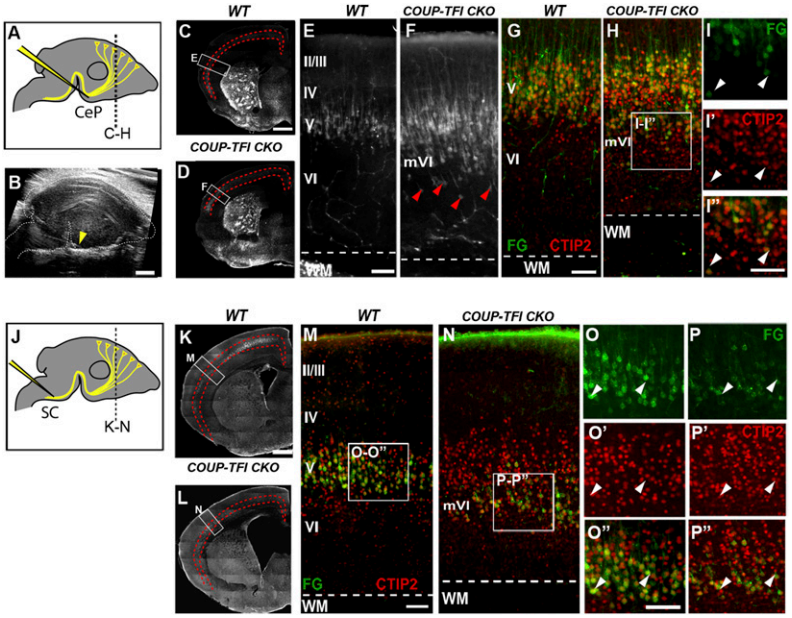

Fig. 3. Corticofugal neurons in superficial layer VI send axons to subcerebral targets in *COUP-TFI CKO* brains. Sagittal schematic views (*A* and *J*) and ultrasonographic image (*B*) of a mouse brain showing FluoroGold (FG) injection in the cerebral peduncle (CeP and *arrowhead* in *B*) and cervical spinal cord (SC). (*C*, *D*, *K*, and *L*) Retrogradely labeled coronal hemisections of P7 brains, with locations of the higher-magnification panels. (*E–I″*) FG injection into the CeP shows abnormally located retrogradely labeled neurons in superficial layer VI (*red arrowheads* in *F*), which are also positive for CTIP2. (*M–P″*) FG injection (*green* in *M*) into the SC labels abnormally located retrogradely labeled neurons exclusively in superficial layer VI. These neurons express CTIP2 (*arrowheads* in *O–P″*). WM, white matter. [Scale bars: 0.5 mm (*B–D, K,* and *L*); 50 μm (*E–H*, *M*, and *N*); 20 μm (*I–I″* and *O–P″*).]

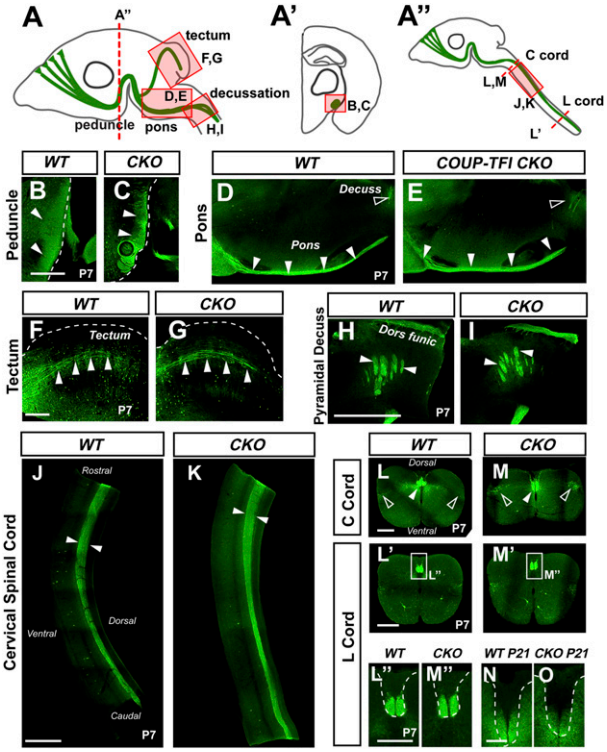

Fig. 4. Cortico-subcerebral connectivity in *COUP-TFI CKO* mice. Schematic sagittal (*A* and *A″*) and coronal (*A′*) views of the brain indicating location of the immunofluorescence photomicrographs shown in *B* to *O* along the corticospinal/corticotectal pathways (*green*) and obtained in *CST-YFP WT* and *COUP-TFI CKO* mice. At P7, corticofugal axons in *WT* and *COUP-TFI CKO* mice fasciculate compactly within the cerebral peduncles (*arrowheads* in *B* and *C*), the pons (*arrowheads* in *D* and *E*), and tectum (*arrowheads* in *F* and *G*), and decussate normally in the medulla oblongata (*H*, *I*, and *open arrowhead* in *D* and *E*). Within the spinal cord (*J–O*), the corticospinal tract is compactly bundled within the dorsal funiculus (Dors funic) in the cervical cord (*J–M*). Even though the CST appears normal at P7 (*L′–M″*), there is a variable defect in the number of lumbar projecting CST axons in *COUP-TFI CKO* mice at P21 (*N*, *O*). [Scale bars: 250 μm (*B–K*); 100 μm (*L–O*).]

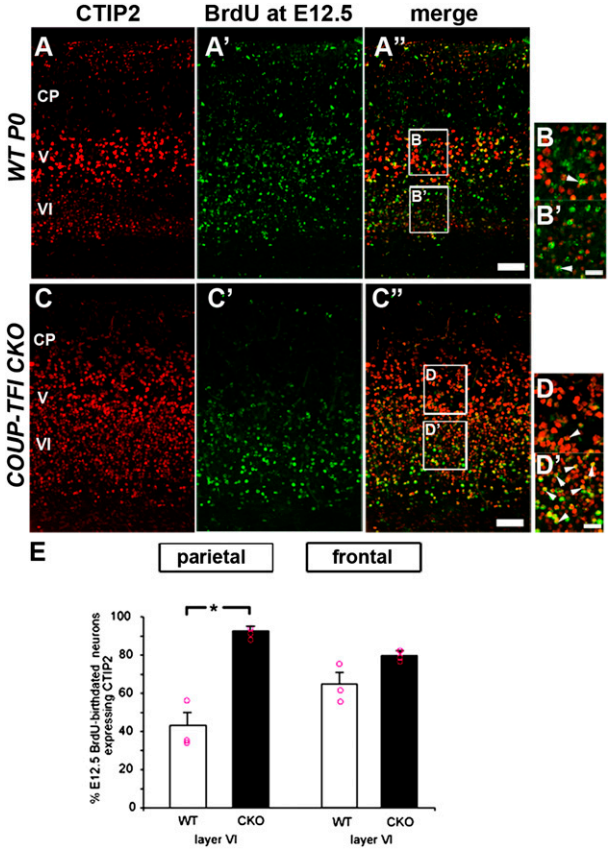

Fig. 5. Loss of COUP-TFI function causes abnormal timing of specification of subcerebral projection neurons in S1 cortex. Coronal sections of P0 *WT* (*A–A″*) and *COUP-TFI CKO* (*C–C″*) parietal cortices after BrdU injection at E12.5, immunostained for BrdU and CTIP2. Higher magnification views of layers V (*B* and *D*) and VI (*B′* and *D′*) indicate a higher number of E12.5 double-labeled neurons in superficial layer VI of *COUP-TFI CKO* brains (*arrowheads* in *D′*). (*E*) Graphical representation of the percentage of BrdU-birthdated neurons expressing high levels of CTIP2 in superficial layer VI of *WT* and *COUP-TFI CKO* parietal and motor cortices. Pink circles indicate values for individual experiments ($n = 3$). CP, cortical plate. [Scale bars: 50 μm (*A–A″* and *C–C″*); 40 μm (*B*, *B′*, *D*, and *D′*).]

superficially located in layer VI) corticothalamic neurons, which are generated immediately before CSMN, raising the possibility that during corticogenesis COUP-TFI acts to control the timing of the transition between corticothalamic and corticospinal motor neuron generation. Thus, we first determined the date of birth of corticofugal neurons in *COUP-TFI CKO* mice by injecting BrdU from E11.5 to E13.5, the normal birth dates of corticofugal neurons (7, 24), and found that the laminar distribution of BrdU birth-dated cells is not distinguishable between *WT* and *COUP-TFI CKO* mice at P0 (Fig. S7). This indicates that both the migration and the survival of corticofugal neurons are unaffected by the absence of COUP-TFI function. Next, we examined whether loss of COUP-TFI increased the probability of E12.5 BrdU birth-dated corticothalamic neurons to strongly express CTIP2, taken as a bona fide index of CSMN differentiation (Fig. 5*A–D′*). Loss of COUP-TFI function leads to a 2-fold increase in the number of E12.5-born neurons that strongly express CTIP2 in layer mVI (Fig. 5*E*) (*WT*, $43 \pm 7\%$; *CKO*, $93 \pm 2\%$; $n = 3$; $P = 0.02$), indicating that COUP-TFI normally acts to restrict CSMN specification during corticothalamic neurogenesis in S1 cortex. Strikingly, this control is exerted in an area-specific manner, because, in frontal/motor cortex, E12.5-born neurons are not more likely to strongly express CTIP2 in the absence of COUP-TFI function (Fig. 5*E*) (*WT*, $65 \pm 6\%$; *CKO*, $83 \pm 4\%$; $n = 3$; $P = 0.07$). Importantly, this area-specific premature generation of CSMN is limited to the time when corticothalamic neurons are being generated (Fig. S8). Together, these data strongly suggest that COUP-TFI normally acts to control the area-specific timing of the transition between corticothalamic and CSMN specification by setting the onset of CSMN differentiation to appropriate time points of corticogenesis in the S1 cortex.

***COUP-TFI CKO* Mice Have Impaired Skilled Motor Behavior.** We next investigated how the increase in motor area size (17), and the reassignment of the corticospinal connectivity to layer VI corticothalamic neurons, might affect sensorimotor function in *COUP-TFI* adult mutant mice. We first investigated sensorimotor function by using an adhesive patch removal task (25), in which the mouse has to remove a piece of adhesive patch placed on each hindpaw. We find that *COUP-TFI CKO* mice are significantly less efficient in removing the patches than *WT* mice (Fig. 6*A*) ($F_{1/19} = 13.635$; $P = 0.001$; see also *SI Methods*). This decreased performance reflects impairment in motor function rather than reduced tactile perception, as *COUP-TFI CKO* mice readily detect the presence of the patch (as indicated by similar latencies in the first attempt to remove the patch) (Fig. 6*B*) ($F_{1/19} = 0.145$; $P = 0.7$). Once the patch is detected, *COUP-TFI CKO* mice make significantly more removal attempts than *WT* mice (Fig. 6*C*) ($F_{1/19} = 21.382$; $P < 0.001$), suggesting specific impairment in fine motor control.

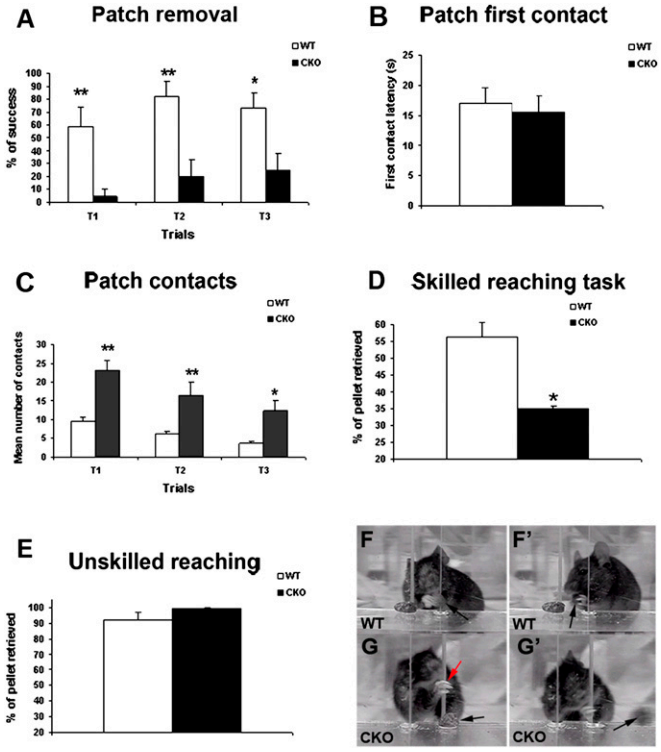

Fig. 6. *COUP-TFI CKO* mice have a specific impairment in corticospinal tract-mediated fine motor skills. (*A*) Decreased success rate in *COUP-TFI CKO* mice in three consecutive trials of the patch removal test, but (*B*) normal latency to first contact with one of the two patches. (*C*) Significantly increased removal attempt numbers, but decreased removal success rate (*A*) reflects normal primary sensory function, but impaired motor function. (*D*) *COUP-TFI CKO* mice have a significantly decreased success rate when compared to *WT* mice in the skilled motor task, but (*E*) have unaffected performances in an unskilled motor task. Photographs of control (*F* and *F'*) and *COUP-TFI CKO* (*G* and *G'*) mice during the skilled reaching task. Control mice grasp foods pellets (location indicated by *black arrows* in *F* to *G'*) with their paws and transfer them to their mouths, whereas *COUP-TFI CKO* mice misguide their paws (*red arrow* in *G*) without grasping food pellets properly (*G'*). Error bars represent SEM. Student's *t* test, $*P \leq 0.01$; $**P \leq 0.001$.

We further examined cortical motor function by employing a task that specifically tests corticospinal tract-mediated motor function, the single pellet skilled reaching task (26, 27). This test assesses the ability of rodents to perform a series of precise skilled movements of their forelimbs to retrieve food through a thin slot. We find that corticospinal motor function is dramatically impaired in *COUP-TFI CKO* mice, as indicated by a strikingly lower rate of pellet recovery compared to *WT* mice (Fig. 6 *D*, *F*–*G'*) ($P = 0.005$) (see also Movie S1). Importantly, however, in a motor task that does not require a forelimb skilled behavior (see also *SI Methods*) (26, 27), *COUP-TFI CKO* mice and *WT* mice are indistinguishable (Fig. 6*E*) ($P = 0.5$), as they are in other behavioral tasks testing more general aspects of motor function, such as muscular strength and pure motor coordination (Fig. S9). These results demonstrate that, even in the presence of relatively preserved corticospinal connectivity, the imprecise areal and temporal specification of CSMN in *COUP-TFI CKO* mice critically impairs the function of the cortical neuronal networks controlling skilled motor behavior.

Discussion

In this study, we show that COUP-TFI plays a critical role in regulating the area-specific balance between corticothalamic neurons and corticospinal motor neurons. In the absence of COUP-TFI function, neurons that, in the S1 cortex, would normally differentiate into corticothalamic neurons, prematurely and abnormally differentiate as CSMN and send their axons to all segmental levels of the spinal cord. Furthermore, genuine CSMN neurons are abnormally differentiated and fail to project to the spinal cord, which results in impaired fine motor skills in *COUP-TFI CKO* adult mice. These data reveal that COUP-TFI exerts an area-specific control over a generic CSMN differentiation program during corticogenesis, and strongly indicate that precision in areal and temporal CSMN differentiation is fundamental for high-level motor function.

COUP-TFI Regulates the Areal- and Temporal-Specific Onset of Corticospinal Motor Neuron Specification. Our study has confirmed that COUP-TFI acts to precisely control the areal and temporal specification of CSMN during corticogenesis, and might, thus, contribute to the larger number of CSMN in motor compared to sensory areas (12). We have shown that loss of COUP-TFI function most severely affects late-born layer VIa corticothalamic neurons, located just below the large-sized pyramidal projection neurons in layer Vb (Fig. S10 *A* and *B*). Our findings suggest that these neurons could be "transitional" forms of corticofugal neurons expressing transiently overlapping gene determinants that normally are under precise molecular and temporal control of COUP-TFI. Interestingly, because corticothalamic neurons still project to distinct thalamic nuclei in the absence of COUP-TFI (17), the abnormal subcerebral projecting neurons in mVI may have thalamic collaterals or, alternatively, distinct populations of subcortical and subcerebral projecting neurons may coexist in this layer.

COUP-TFI Is a Negative Regulator of a Genetic CSMN Differentiation Program. Several studies have demonstrated that the fate specification and differentiation of CSMN are under the control of a combinatorial set of transcription factors that can either induce or inhibit CSMN-specific genes during corticogenesis (8, 9, 21, 28, 29). In the present study, we provide evidence that COUP-TFI is an upstream negative regulator of a CSMN differentiation program in corticofugal neurons, through repression of a genetic program of CSMN differentiation. First, during the period of corticofugal neuron birth and specification, an increased number of *Fezf2*- and CTIP2-positive neurons in *COUP-TFI CKO* mice are generated; second, COUP-TFI-deficient neurons in layers V and VI express abnormally high levels of *Fezf2*, CTIP2, *Crim1*, and *Igfbp4*; third, gain-of-function of COUP-TFI in a transgenic model shows decreased expression of *Fezf2* and other layer V markers (19). An especially interesting aspect of these results is the abnormal differentiation of genuine CSMN in layer V of *COUP-TFI CKO* mice, including failure of these neurons to send projections to the spinal cord. It is likely that abnormal expression of corticothalamic neuron genes during CSMN differentiation, and dysregulation of CSMN "control genes" centrally contribute to improper differentiation of these neurons. In support of this latter hypothesis, ectopic expression of *Fezf2* and CTIP2 leads to altered axonal trajectories (6, 28), and dose-dependant effects of CTIP2 on CSMN differentiation have been reported (4, 8). indicating that precise levels of CSMN-specific genes are a fundamental requisite for correct differentiation of corticofugal neurons. Finally, because COUP-TFI and *Fezf2* are both expressed in postmitotic neurons, it is possible that abnormal differentiation of CSMN and corticothalamic neurons in the absence of *COUP-TFI* occurs at a postmitotic level, as recently shown for other cortical transcriptional regulators (8, 30, 31, 32).

COUP-TFI-Dependent Precision of Corticospinal Neuron Differentiation Is Critical for Skilled Motor Behavior. In the present study, we found that, in the absence of *COUP-TFI* skilled motor function related to the sensorimotor cortex and corticospinal motor tract is substantially impaired. These behavioral data indicate that precise complement and connectivity of the distinct corticofugal neuronal populations is critical for proper motor function (27, 33). Genuine

CSMN in layer V do not project to the spinal cord in *COUP-TFI CKO* mice; however, these mice remarkably establish corticospinal projections to all segments of the spinal cord. Our retrograde tracings from the spinal cord demonstrate that the tract originates largely from the corticothalamic neurons in layer mVI within the abnormally expanded motorized cortex (17). Thus, these misspecifed neurons may not be integrated into appropriate cortical motor neuronal networks, and therefore are unable to contribute to fine motor control. Interestingly, *COUP-TFI CKO* mice largely reproduce behavioral defects observed in rats after either specific corticospinal lesions or motor or somatosensory cortical ischemia (26, 27, 33, 34), emphasizing that dysregulation of precise CSMN development leads to deficits of high-level motor function and behavior.

Methods

Mice. *COUP-TFI CKO* mice were generated and genotyped as shown previously (17). For genetic labeling of the entire corticospinal motor tract, *COUP-TFI CKO* mice were crossed with *Thy1-STOP-YFP* mouse (23) (kind gift of J. Sanes). All experiments were conducted following guidelines of the Institutional Animal Care and Use Committee, Cardarelli Hospital, Naples, Italy, and in accordance with institutional and federal guidelines of the Massachusetts General Hospital IACUC.

Immunocytochemistry, In Situ Hybridization, and Histology. Brains were treated and processed for free-floating and standard immunofluorescence protocols as described (4, 17). YFP detection was amplified with a GFP-specific antibody (1:1000 Chemicon). Whole-mount in situ hybridization and nonradioactive in situ hybridization were performed as described (17). Antisense RNA probes were labeled using a DIG-RNA labeling kit (Roche). For Nissl staining, sections were stained with 0.5% cresyl violet, as described (4).

Retrograde Labeling. Subcerebrally projecting neurons were retrogradely labeled via FluoroGold injections into the cerebral peduncle or spinal cord, at P2 and P3 under ultrasound guidance (Vevo 660, VisualSonics), as described (4). Injected mice were collected at P6 or P7 and processed for immunocytochemistry. Each experiment was repeated at least three times and showed reproducible results.

BrdU Birth Dating. Timed pregnant females received a single i.p. injection of BrdU (50 mg/kg) at E11.5, E12.5, E13.5, or E15.5. Pups were collected at birth, processed for BrdU immunocytochemistry, and quantified as described (6).

Behavioral Analysis. For the adhesive patch removal task, an adhesive patch was placed on the dorsal surface of each hindpaw, then mice were released in the testing cage and observed for 240 s. Animals underwent three consecutive trials, with an intertrial interval of 60 min. The skilled reaching task was adapted in mice from studies in rats (27). The task consisted of three phases: habituation, unskilled reaching, and skilled reaching. More details of both tasks are available in *SI Methods*.

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